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Developmental Biology

# Developmental gene expression and tissue distribution of the CHIP28 water-channel protein

(*in situ* hybridization/fetal development/red cell/kidney/choroid plexus)

CAROLYN BONDY*, EDWARD CHIN*, BARBARA L. SMITH†, GREGORY M. PRESTON†, AND PETER AGRE†‡

*Developmental Endocrinology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; and †Departments of Medicine and Cell Biology/Anatomy, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205

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**ABSTRACT** **The CHIP28 water channel is a major component of red cell and renal tubule membranes; however, its ontogeny and tissue distribution remain undefined. Three patterns of expression were identified when CHIP28 mRNA was surveyed by *in situ* hybridization histochemistry in rats between embryonic day 14 and maturity. (*i*) CHIP28 mRNA and protein were very abundant in hematopoietic tissue and kidneys of mature rats, but strong expression did not occur until after birth, when it appeared in renal proximal tubules and descending thin limbs, red pulp of the spleen, and membranes of circulating red cells. (*ii*) CHIP28 mRNA was abundant in choroid plexus epithelium throughout fetal development and maturity. (*iii*) CHIP28 mRNA was transiently observed in periosteum, heart, vascular endothelium, and cornea during fetal development. The ontogeny of kidney and red cell CHIP28 expression coincides with the ability of kidneys to concentrate urine, suggesting that CHIP28 promotes water reabsorption in the proximal nephron and provides red cell osmoregulation needed for passage through the hypertonic medulla. Its presence in the choroid plexus suggests that CHIP28-mediated water transport contributes to secretion of cerebrospinal fluid. The functional role of CHIP28 in developing bone, heart, and eye is unclear. These findings further establish the general physiologic role of CHIP28 as a water channel involved in reabsorption, osmoregulation, and secretion. The studies also suggest other possible functions during fetal development and predict that complex mechanisms will be needed for regulation of CHIP28 gene expression in diverse tissues at distinct points in development.**

The plasma membranes of most cells exhibit some water permeability due to diffusion through the lipid bilayer; the plasma membranes of red cells, renal tubules, and certain other epithelial cells exhibit high water permeability which has been predicted to result from water-selective channels (1). At least two forms of water channel activities have been identified: constitutively active water channels in red cells and renal proximal tubules, and vasopressin-regulated water channels in the renal collecting ducts (2, 3).

The first water-channel protein identified, CHIP28, is a 28-kDa integral membrane protein that is abundant in red cells and proximal renal tubules (4, 5) and resembles tetrameric membrane channel proteins (6). The deduced primary amino acid sequence of CHIP28 (7) is related to MIP26, major intrinsic protein of lens, and to other members of a newly recognized family of membrane proteins from diverse organisms (8). CHIP28-mediated water channel activity was first demonstrated by expression in *Xenopus* oocytes (9), and the selectivity and unit water permeability coefficient were determined with proteoliposomes reconstituted with highly purified CHIP28 protein (10).

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The physiological need for water-selective channels in the membranes of red blood cells is not understood. It is not known in which other tissues CHIP28 may be expressed. In addition to renal tubules, the membranes of several other epithelia are highly permeable to water and may contain CHIP28 water channels or homologous proteins. Consistent with this, CHIP28 mRNA was recently identified by Northern analysis of several organs from mouse, and the deduced amino acid sequence of mouse CHIP28 was 94% identical to the human CHIP28 sequence (11). *In situ* hybridization histochemistry was therefore undertaken in sections of fetal, postnatal, and mature rats to identify the tissue locations of CHIP28 mRNA and to define the points in development where it is expressed.

## MATERIALS AND METHODS

***In Situ* Hybridization Histochemistry.** Timed pregnant Sprague–Dawley rats were obtained from Taconic Farms, Germantown, NY. Fetal rats were studied at 14, 15, 18, and 20 days of gestation; pups were studied 5, 16, 20, and 40 days after birth. Animals were anesthetized by $CO_2$ inhalation and decapitated in accord with protocols approved by the National Institute of Child Health and Human Development and the Johns Hopkins University. Tissues were frozen in dry ice and sectioned at −15°C to a thickness of 10 μm. Sections were thaw-mounted onto poly(L-lysine)-coated slides and stored frozen until use. Spleens were obtained from mice recovering from thiamphenicol-induced anemia (12).

Procedures for synthesis of RNA probes and *in situ* hybridization were described in detail (13). An exonuclease III fragment corresponding to nucleotides +15 to +451 of the human CHIP28 cDNA was subcloned into pBluescript II (Stratagene) for transcription of both antisense (using T3 RNA polymerase) and sense (using T7 RNA polymerase) RNA probes. After hybridization, sections were exposed to autoradiographic film (Hyperfilm-βmax, Amersham) for 5–7 days and were later dipped in photographic emulsion (NTB3, Kodak) for 20 days. Sense control probe hybridizations of parallel tissue sections exposed for the same length of time produced negligible signal (data not shown).

**Western and Northern Blots.** Red cells were harvested from fetal, postnatal, and mature rats; membranes were analyzed by SDS/PAGE, and Western immunoblots were probed as described with affinity-purified CHIP28 antibodies (6) or affinity-purified antibodies specific for human ankyrin and band 3 (obtained from Vann Bennett, Duke University Medical Center). Northern analysis of tissue mRNA was performed as described (7) with a Micro-FastTrack kit (Invitrogen, San Diego).

Abbreviations: E*n*, embryonic day *n*; P*n*, postnatal day *n*.

‡To whom reprint requests should be addressed.

## RESULTS

**Postnatal Expression in Erythroid and Renal Tissues.** RNAs from bone marrow, spleen, kidney, and several other organs from mature rats and mice demonstrated a single 2.9- to 3.0-kb transcript on Northern blots probed with CHIP28 cDNA, but the tissue distribution of the transcript within these organs is not known (7, 11). Since the CHIP28 water-channel protein is highly abundant in the membranes of red cells and renal tubular epithelia in mature animals (4–6), it was surprising that 1 day before birth (embryonic day 20, E20), *in situ* hybridizations with a CHIP28 RNA probe revealed virtually no signal over kidney, liver, or spleen (Fig. 1 *A* and *B*) or over nucleated red cells. The lack of hybridization signal over fetal erythroid tissues did not reflect a technical difficulty, since *in situ* hybridizations revealed marked hybridization signal over regions of erythroid production in mature, anemic mouse spleen (Fig. 1 *C–F*).

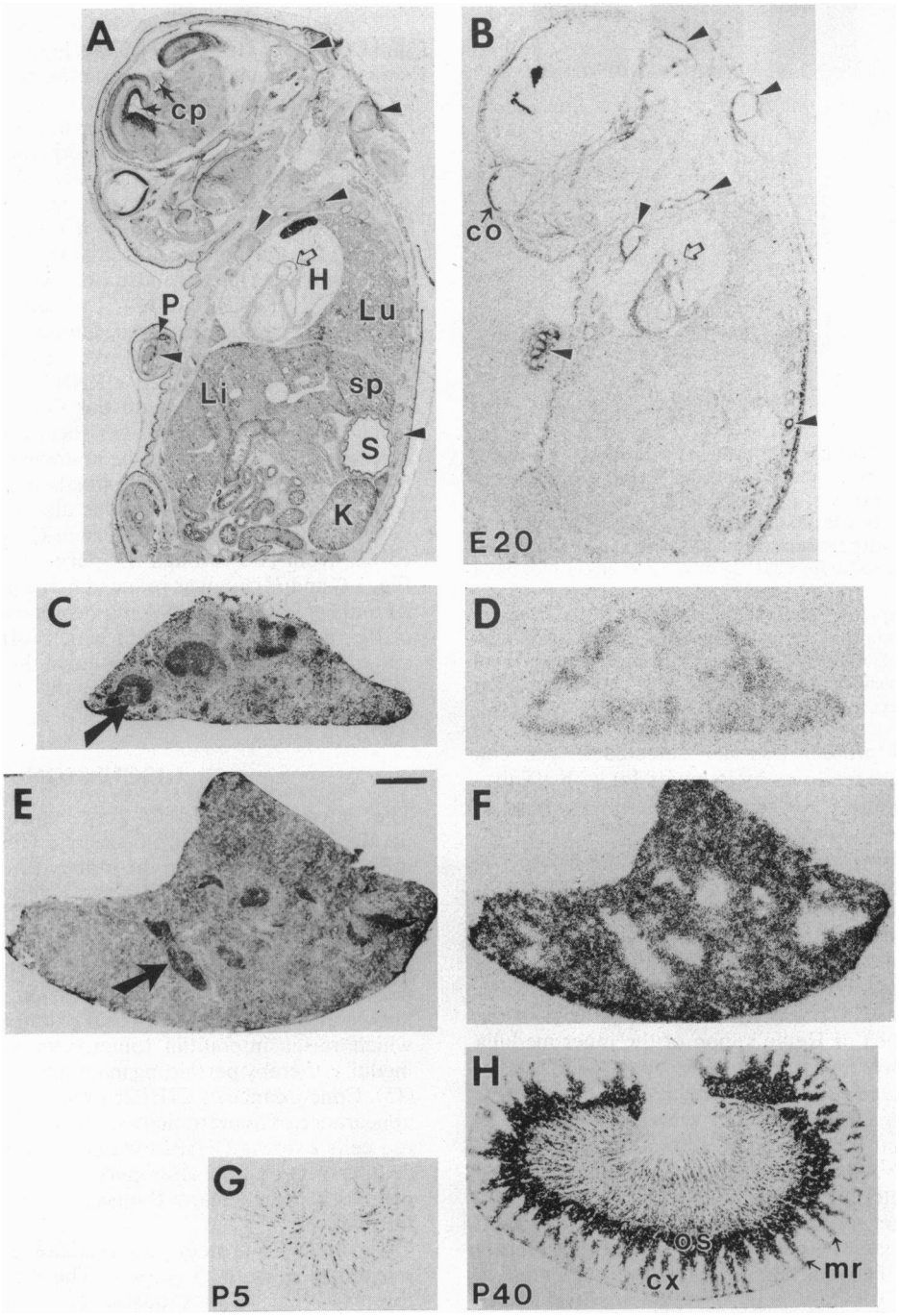


FIG. 1. Tissue distribution of CHIP28 gene expression shown by film autoradiography. (*A* and *B*) Sagittal section from an E20 rat fetus after staining with hematoxylin and eosin (*A*) and after hybridization to the CHIP28 RNA probe and visualization by autoradiography (*B*). Tissue symbols: co, cornea; cp, choroid plexus; H, heart; K, kidney; Li, liver; Lu, lung; P, forepaw; S, stomach; and sp, spleen. Arrowheads identify periostium surrounding bone anlage. The open arrow indicates the aortic valve where leaflets are intensely positive for CHIP28 mRNA. (*C–F*) Spleens from mature, normal mice (*C* and *D*) and anemic littermates (*E* and *F*). *C* and *E* are stained tissue sections; *D* and *F* are corresponding film autoradiographs which reveal CHIP28 mRNA concentrated over red pulp but not lymphoid nodules (arrows). (*G* and *H*) CHIP28 gene expression in rat kidneys at P5 and P40. Sections in *C–H* were hybridized in the same experiment and exposed to the same piece of film to permit comparison of hybridization signal intensities. Tissue symbols: cx, cortex; mr, medullary ray; os, outer stripe of the outer medulla. (Bar = 2.7 mm in *A* and *B*, 0.8 mm in *C–F*, and 2 mm in *G* and *H*.)

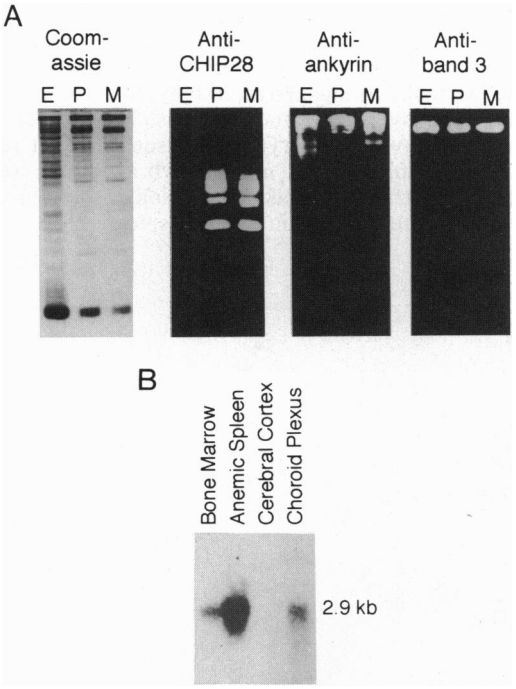


FIG. 2. Blot analyses of CHIP28 protein and mRNA. (*A*) Immunoblot of red cell membranes obtained from E18 (lanes E), P16 (lanes P), and mature (lanes M) rats. Approximately 5 μg of protein was electrophoresed into sodium dodecyl sulfate/12% polyacrylamide gels and then stained with Coomassie blue or analyzed by immunoblot with antibodies specific for CHIP28 or red cell ankyrin or band 3. (*B*) Northern blot of total RNA from human bone marrow (15 μg) and anemic mouse spleen (40 μg) and poly(A)$^+$ RNA from rat cerebrum (2 μg) and choroid plexus (1 μg) after hybridization with $^{32}$P-labeled CHIP28 cDNA (9). Radiographic exposure times were adjusted to improve visualizations: bone marrow and spleen, 8 hr; brain and choroid plexus, 24 hr.

Likewise, CHIP28 protein was not detected in red cell membranes from E18 fetal rats by immunoblot with affinity-purified polyclonal anti-CHIP28, whereas ankyrin, band 3, and spectrin were clearly present (Fig. 2*A*).

Expression of CHIP28 in red cells and kidney was induced soon after birth. Immunoblots of red cell membranes from rat pups at postnatal day 16 (P16) were virtually indistinguishable from those of mature rats (Fig. 2*A*). Little CHIP28 mRNA was detected in the kidney up to P5, but levels increased steadily thereafter and stabilized by P40 (Fig. 1 *B*, *G*, and *H*). CHIP28 mRNA was discretely localized in epithelia in the descending thin limbs of Henle's loop of the inner medulla and in the straight proximal tubules of the outer medulla (Fig. 3 *A* and *B*), structures known to exhibit high constitutive water permeability. CHIP28 mRNA was less abundant in convoluted proximal tubules in the cortical labyrinth (Fig. 1*H*). CHIP28 mRNA was not detected in ascending thin limbs, which are water impermeable, or collecting ducts (Fig. 3 *A* and *B*), even after saline or vasopressin treatment, which confers water permeability (data not shown). The distribution of CHIP28 mRNA in kidney is consistent with the known water permeability of these nephron segments and agrees with immunohistochemical staining with anti-CHIP28 IgG (5). This suggests that CHIP28 protein is the constitutive water channel of the proximal nephron but not the vasopressin-regulated water channel of the collecting duct (14).

**Fetal and Postnatal Expression in Choroid Plexus.** CHIP28 mRNA was highly abundant in choroid plexus epithelium but was not detected elsewhere in brain. The transcript was observed during fetal development (Figs. 1*B* and 3*C*) and persisted in the choroid plexus of mature animals (Fig. 2*B*). The epithelial cells of the choroid plexus secrete cerebrospinal fluid by active transport of $Na^+$ into the ventricular space, which evokes the osmotic movement of water in the same direction. The intense hybridization signal localized at this site strongly suggests that CHIP28-mediated water flow is necessary for secretion of cerebrospinal fluid. Consistent with this, CHIP28 protein has been localized immunohistochemically in the apical membrane of choroid plexus epithelium of mature rat (S. Nielsen and P.A., unpublished work).

**Transient Prenatal Expression in Periostium, Endocardium, and Cornea.** CHIP28 mRNA was highly abundant in condensations of mesenchyme forming the periostium around sites of endochondral and intramembranous ossification (Figs. 1*B* and 3*D*). The periostium is formed by a collaboration of osteoblasts, fibroblasts, and endothelial cells, and it was not possible to resolve which of these cell types expressed the transcript. CHIP28 mRNA was also observed in the fetal heart from E14 to E16, where it was most abundant in the endocardial cushions, primordial valves, and septa, but low levels of transcript were also observed throughout the myocardium (Figs. 1*B* and 3*E*). Cardiac CHIP28 mRNA was greatly decreased after birth, although it was still diffusely detectable throughout the myocardium and endocardium (data not shown). CHIP28 mRNA was also identified in a serpiginous pattern beneath the skin during fetal development (Fig. 1*B*), where it was localized in endothelium of dermal vessels (Fig. 3*F*). The abundance was greatly diminished in the skin of mature animals (data not shown).

CHIP28 gene expression was also prominent during eye development. Abundant CHIP28 mRNA was observed in the cornea from E15 through the perinatal period (Fig. 1*B* and Fig. 3 *G* and *H*) but was reduced in the postnatal cornea (Fig. 3 *I* and *J*). CHIP28 mRNA was also observed in that portion of the optic neuroepithelium which forms ciliary apparatus and iris. CHIP28 mRNA persisted in the posterior epithelium of the iris but was reduced in the ciliary process in the postnatal eye (Fig. 3 *I* and *J*).

## DISCUSSION

The ontogeny of CHIP28 gene expression in the kidney paralleled that in erythroid tissues. Only traces of CHIP28 mRNA were detected in the kidney prior to birth and in the early postnatal period; peak levels were not seen until about 3 weeks after birth. The lack of CHIP28 in fetal red cells demonstrates that the protein is not essential for oxygen delivery or survival within the fetal circulation. The timing of peak renal CHIP28 gene expression coincided with the known development of the renal countercurrent mechanism, which raises interstitial tonicity above 1 M in the renal medulla, thereby permitting maximum concentration of urine (15). Concordance of CHIP28 expression in red cells and the appearance of hypertonicity in the renal medulla implies that red cells express CHIP28 water channels to facilitate rapid cellular rehydration after permeating the renal medulla, a process repeated many thousand times during the red cell lifespan.

The CHIP28 protein may mediate transmembrane water movement in both directions. The direction of water flow through renal tubules during reabsorption of glomerular filtrate, from tubular lumen to plasma, is opposite to the direction of flow through the choroid plexus during secretion of cerebrospinal fluid, from plasma to ventricular space. In contrast, the osmoregulatory mechanism in red cells would require CHIP28-mediated water movements in either direction, depending upon the external osmolality. The proposed roles of CHIP28 protein as the channel facilitating water reabsorption, secretion, and bidirectional osmoregulation is consistent with the 180° axis of symmetry separating the two

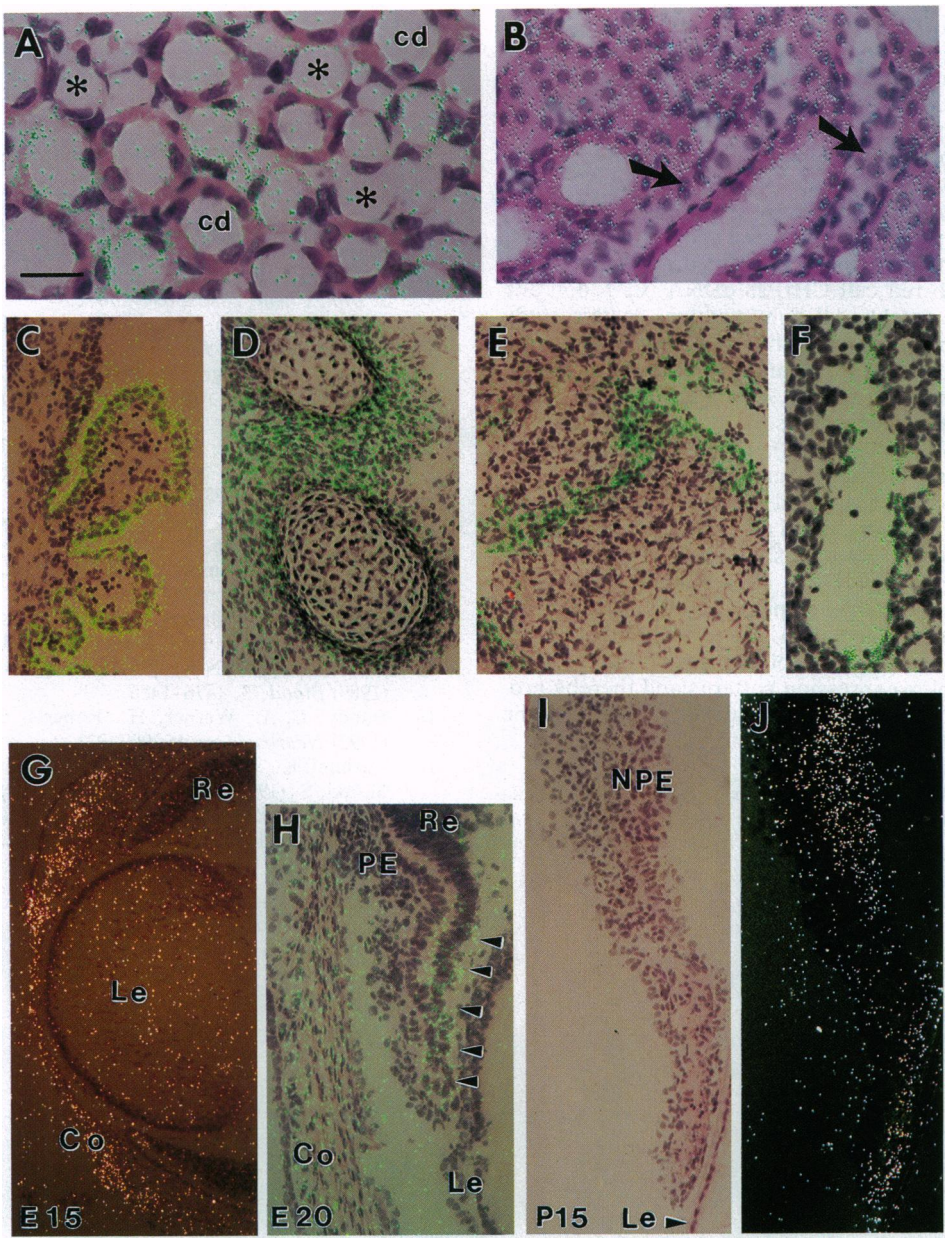


FIG. 3. Microscopic CHIP28 mRNA localization by nuclear emulsion autoradiography. (*A* and *B*) Mature rat kidney. The inner medulla (*A*) contains ascending (*) and descending thin limbs of Henle's loop and collecting ducts (cd). CHIP28 mRNA (green grains) is detected only in descending thin limbs. In the outer stripe of the outer medulla (*B*), CHIP28 mRNA is abundant in the straight proximal tubules but is not detected in distal nephron or collecting duct (arrows). (*C–G*) E15 rat. CHIP28 mRNA is localized in the epithelial layer of the choroid plexus (*C*). Mesenchyme surrounding cartilage anlage of ribs contains abundant CHIP28 mRNA (*D*). The atrioventricular cushions and outflow tract endocardium show strong hybridization (*E*). Endothelial cells in a dermal vein contain CHIP28 mRNA (*F*). (*G–J*) CHIP28 mRNA in the developing eye from E15 to P15. A darkfield view reveals hybridization signal (white grains) concentrated in the developing cornea (*G*). Hybridization (green grains) is observed in the cornea (Co) and in an outgrowth of the nonpigmented retinal epithelium (row of arrowheads); hybridization was not detected in the lens (Le), retina (Re), or pigmented epithelium (PE) (*H*). Paired bright- and darkfield images demonstrate CHIP28 mRNA confined to the nonpigmented or posterior epithelium (NPE) of the iris in the postnatal eye (*I* and *J*). (Bar = 12 μm in *A* and *B*, 25 μm in *C–F*, and 50 μm in *G–J*.)

internal repeats within the predicted structure of the CHIP28 protein (7, 9). This suggests that CHIP28 protein may have evolved to facilitate reciprocal movements of water across the plasma membrane into or out of a cell, flowing in the direction of the higher osmolality.

The physiologic importance of transient expression of CHIP28 mRNA in periostium, endocardium, and cornea is not obvious. CHIP28 mRNA was recently identified among the delayed early response gene mRNAs in fibroblasts which were transcribed 6–8 hr after stimulation with growth factors and were degraded before DNA synthesis (11). This pattern resembles the transient expression of CHIP28 mRNA in fetal periostium, suggesting a need for CHIP28 in connective tissue during growth and development. This pattern also resembles the transient expression of CHIP28 mRNA, which coincides with vascularization of the fetal cornea. Very pronounced changes in hydration occur in the corneal stroma at this time, resulting in corneal swelling and subsequent dehydration (16). A common thread among diverse settings of transient CHIP28 gene expression during fetal development is the association with endothelial cells and extracellular matrix deposition, a feature of CHIP28 gene expression observed during development of the cardiac septae, valves and outflow tracts, periostium, and cornea. How CHIP28

might be involved in the formation of these dense connective tissue structures is unclear, but it may mediate major changes in hydration accompanying the deposition of these extracellular matrix components.

These studies predict that CHIP28 gene expression is under complex controls which provide signals for biosynthesis of the protein in diverse tissues at distinct points in development. Although not formally proven, it is most likely that the same gene product is expressed in all of these tissues. The cDNA from human kidney contained a nucleotide sequence identical to red cell CHIP28 cDNA (C. Moon and P.A., unpublished work). All hybridizations in this study were high stringency, and cross hybridization was not observed over posterior lens, the site of MIP26, a homologous gene product. Moreover, only 2.9-kb transcripts were observed by Northern analysis of several tissues (7, 11), including choroid plexus (Fig. 2*B*). Immunoblot studies of several perfused tissues from mature rats including red cells, kidney, choroid plexus, heart, and cornea revealed identical 28-kDa membrane proteins which reacted with anti-CHIP28 IgG (S. Nielsen and P.A., unpublished work). The molecular mechanisms controlling CHIP28 expression remain to be identified but may involve developmentally coordinated sets of transcription factors. Isolation of the CHIP28 structural gene may permit identification of the molecular elements behind these complex expression patterns and thereby provide insight into basic mammalian development and the physiologic needs for the CHIP28 water channel.

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